

Running title: CSF and genetic factors in MS

Genetic variants are major determinants of cerebrospinal fluid antibody levels in multiple sclerosis

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Abstract

Immunological hallmarks of multiple sclerosis include the production of antibodies in the central nervous system, expressed as presence of oligoclonal bands and/or an increased Immunoglobulin G index, the level of immunoglobulin G in the cerebrospinal fluid compared to serum. However, the underlying differences between oligoclonal band positive and negative MS patients and reasons for variability in Immunoglobulin G index are not known. In order to identify genetic factors influencing the variation in the antibody levels in the cerebrospinal fluid in multiple sclerosis, we have performed a genome-wide association screen in patients collected from nine countries for two traits, presence or absence of oligoclonal bands (n=3,026) and Immunoglobulin G index levels (n=938), followed by a replication in 3,891 additional patients. We replicate previously suggested association signals for oligoclonal band status in the Major Histocompatibility Complex region for the rs9271640*A-rs6457617*G haplotype, correlated with HLA-DRB1*1501, and rs34083746*G, correlated with HLA-DQA1*0301 (P comparing two haplotypes = $8.88\text{E-}16$). Furthermore, we identify a novel association signal of rs9807334, near the *ELAC1/SMAD4* genes, for oligoclonal band status ($P = 8.45\text{E-}07$). The previously reported association of the Immunoglobulin Heavy Chain locus with Immunoglobulin G index reaches strong evidence for association in this dataset ($P = 3.79\text{E-}37$). We identify two novel associations in the Major Histocompatibility Complex region with Immunoglobulin G index: the rs9271640*A-rs6457617*G haplotype ($P = 1.59\text{E-}22$), shared with oligoclonal band status, and an additional independent effect of rs6457617*G ($P = 3.68\text{E-}06$). Variants identified in this study account for up to two-fold differences in the odds of being oligoclonal band positive and 7.75 % of the variation in Immunoglobulin G index. Both traits are associated with clinical features of disease such as female gender, age at onset and severity. This is the largest study population so far investigated for the genetic influence on antibody levels in the cerebrospinal fluid in multiple sclerosis, including 6,950 patients. We confirm that genetic factors underlie these antibody levels and identify both the Major Histocompatibility Complex and Immunoglobulin Heavy Chain region as major determinants.

Keywords:

multiple sclerosis, cerebrospinal fluid, oligoclonal bands, immunoglobulin, genetics

Abbreviations:

AAO	Age at onset
HLA	Human Leukocyte Antigen
IgG	Immunoglobulin G
MHC	Major Histocompatibility Complex
MSSS	Multiple Sclerosis Severity Score
OCB	oligoclonal band
OR	Odds Ratio
PP	Primary Progressive
SNP	Single Nucleotide Polymorphism

Introduction

Multiple sclerosis is a neurological disease characterized by inflammation, demyelination and axonal degeneration, and is an important cause of disability in young adults (Compston and Coles, 2008). The etiology is not known, but both genetic and environmental factors influence disease susceptibility (Compston and Coles, 2008). An association between multiple sclerosis and the Human Leukocyte Antigen (HLA) genes in the Major Histocompatibility Complex (MHC) region has been identified early on (Jersild *et al.*, 1972), and has later been refined to four independent association signals of which HLA-DRB1*15:01 is the strongest (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). Recently, knowledge of non-HLA associations in multiple sclerosis has increased extensively and immunologically relevant genes are overrepresented amongst those mapping close to the identified risk variants (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011, The International Multiple Sclerosis Genetics Consortium, 2013).

An immunological hallmark in multiple sclerosis is the finding of oligoclonal bands (OCBs) and/or increased Immunoglobulin G (IgG) index in the cerebrospinal fluid (Stangel *et al.*, 2013). OCBs are reported to be observed in 90-95 % of patients in Northern Europe, and are composed predominantly of IgG. The IgG index contrasts the cerebrospinal fluid/serum IgG ratio to the cerebrospinal fluid/serum albumin ratio (Link and Huang, 2006). There are strong indications that antibody levels in the cerebrospinal fluid are influenced by genetic factors, since cerebrospinal fluid abnormalities are seen in 19% of unaffected siblings of multiple sclerosis patients as opposed to 4% of healthy unrelated individuals (Haghighi *et al.*, 2000). Also, the OCB positive rates, as well as the IgG index are reported to correlate with ethnicity (Fukazawa *et al.*, 1998, Kikuchi *et al.*, 2003, Rinker *et al.*, 2007, Lechner-Scott *et al.*, 2011, Yoshimura *et al.*, 2014), although not observed in all studies (Berg-Hansen *et al.*, 2013).

The role of the HLA loci in determining OCB status has been highlighted previously (Fukazawa *et al.*, 1998, Kikuchi *et al.*, 2003, Imrell *et al.*, 2006, Idiman *et al.*, 2009, Wu *et al.*, 2009, Romero-Pinel *et al.*, 2011, Leone *et al.*, 2013, Mero *et al.*, 2013, Yoshimura *et al.*, 2014). Most recently, large study populations from Scandinavia and Italy have shown HLA-DRB1*15:01 to be associated with OCB positive and HLA-DRB1*04:04 with OCB negative multiple sclerosis, respectively (Leone *et al.*, 2013, Mero *et al.*, 2013). Furthermore, the immunoglobulin heavy chain (*IGHC*) region was recently reported to be highly correlated with IgG index in German and Belgian multiple sclerosis patients (Buck *et al.*, 2012).

In the present study we aim to further determine the genetic impact on IgG levels in the cerebrospinal fluid in multiple sclerosis. We combine genome-wide single nucleotide polymorphism (SNP) data (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) with available cerebrospinal fluid data in a meta-analysis of 3,059 multiple sclerosis patients and replicate top-hits from the screening phase in 3,891 additional samples. The combined dataset of 6,950 patients from nine countries is the largest study population so far to investigate both clinical and genetic factors associated with OCB status and IgG index and we replicate three previously described associations and identify three new genetic differences underlying cerebrospinal fluid phenotypes.

Methods

Study populations

The 3,059 multiple sclerosis samples included in the screening phase of this study are a subset of the samples that were previously used in a genome-wide association study on multiple sclerosis susceptibility (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). Eight countries provided OCB status (positive or negative) for 3,026 patients in the screening phase, while five countries provided IgG index for 938 patients (Table 1). In the replication phase, 3,891 additional multiple sclerosis samples (3,842 with OCB status and 2,188 with IgG index) were provided by eight countries. The combined screening and replication cohort included OCB status on 6,868 patients and IgG index on 3,126 patients from the following countries: Australia, Belgium, Denmark, Germany, Italy, Norway, Spain, Sweden and USA (Table 1). For a total of 3,044 patients, both OCB status and IgG index were available. For the genetic analyses, 3,478 patients with OCB status and 2,072 patients with IgG index survived genetic quality control in the replication phase (see further), leading to a final sample size of 6,504 for OCB status and 3,010 for IgG index in the combined genetic analyses.

Cerebrospinal fluid analyses

All patients included in this study fulfilled Poser or McDonald multiple sclerosis criteria (Poser *et al.*, 1983, McDonald *et al.*, 2001) or were diagnosed as clinically isolated syndrome ($n = 7$ (0.2%) in screening phase (Germany); $n = 138$ (3.5%) in replication phase (Germany)), based on the combination of (1) a clinical symptom being typical and suggestive for multiple sclerosis, (2) a magnetic resonance imaging of the brain and in most cases also of the spinal cord demonstrating typical multiple sclerosis lesions fulfilling the criteria of dissemination in space (Barkhof criteria or Swanton criteria) and (3) cerebrospinal fluid parameters. In most countries recruiting samples to this study, lumbar puncture is done routinely as part of the diagnostic process and is seldom repeated unless the diagnosis is unclear. OCB status is considered positive when more than one OCB was seen in the cerebrospinal fluid that was not present in the serum. IgG index is considered positive for values >0.7 . In this study the IgG index value was used in quantitative trait statistical analyses after transforming the IgG index ratio by taking the logarithm (base 2) (\log_2 IgG).

Clinical analyses

The clinical parameters gender, age at onset (AAO), multiple sclerosis severity score (MSSS) (Roxburgh *et al.*, 2005), disease course and disease duration at lumbar puncture were included in the clinical analyses. Statistical analyses of cerebrospinal fluid phenotypes related to demographic and clinical parameters were done in R 2.14.1 (www.r-project.org) using linear regression for IgG index and logistic regression for the binary OCB status. We included covariates in the analyses as indicated.

Screening phase

Quality control and analysis of genetic data provided in the multiple sclerosis genome-wide association study (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) were performed with Plink v1.07 (Purcell *et al.*, 2007). For OCB status, we used a qualitative trait analysis in a logistic regression with binary outcome (OCB pos/neg). For IgG index, we applied a quantitative trait analysis in a linear regression with allele dosage as independent variable and IgG index as dependent outcome. In the screening phase, gender and the seven main principal components previously determined in this dataset (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) were associated with IgG index and hence

added as covariates for IgG index analyses. Evidence of association was tested in samples from each country individually and combined in a fixed-effects meta-analysis (assuming a qualitative trait for OCB and quantitative trait for IgG) over all countries. P -values $< 5E-08$ and $< E-4.5$ in the screening phase were considered as genome-wide significant and suggestive evidence, respectively, as applied previously (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). For each lead SNP a conditional analysis of the surrounding 3 Mb region was performed, and SNPs reaching $P < E-04$ were considered suggestive in this analysis.

Power for OCB analysis was determined mainly by the typical frequency of OCB negative status in a multiple sclerosis study population. In the screening phase, we had 80% power to detect suggestive evidence ($P < E-4.5$) for variants with a minor allele frequency of 0.20 and an odds ratio of 1.6. Power was 80% for variants explaining 2.5% of the variation in the distribution of IgG index seen in multiple sclerosis patients.

Replication phase

In total, 42 SNPs were selected for replication. Of these, 38 SNPs were brought forward to replication based on the results from the screening phase; 32 lead SNPs reaching $P < E-4.5$, an additional proxy marker for rs6457617 (rs9275224 with $r^2 = 1$), and five SNPs with conditional association signals of $P < E-04$. Additionally, we added two SNPs that were previously suggested to be associated with OCB status (Leone *et al.*, 2013, Mero *et al.*, 2013) and two SNPs reported to be associated to autoantibody presence in other tissues (Ferreira *et al.*, 2010, Plagnol *et al.*, 2011) and showing association or a trend for association with IgG index in our study. The SNPs were genotyped using Sequenom MassArray iPLEX technology (Sequenom Inc, San Diego, CA). Sequenom MassArray Designer software v3.1 was used to design primers and extension probes. As part of validating the design, a test set of genotyping data from 86 individuals generated with Illumina platforms was included. Seven SNPs were replaced by proxies with moderate to high linkage disequilibrium ($r^2 > 0.63$). For two SNPs, no assay could be designed and for two others genotyping assays failed, therefore a total of 38 SNPs were successfully included in the replication analysis (i.e. 28 lead SNPs and one synonym, five conditional SNPs and four SNPs selected from previous studies) (Supplementary Material). 75 ng of genomic DNA was used in five μ l reactions in 384-well plates. The amplified resin-treated DNA was spotted with a Sequenom MassArray Nanodispenser (Sequenom Inc, San Diego, CA) on a SpectroCHIP Array. The SpectroCHIP Arrays were analyzed using a Sequenom MALDI-TOF mass spectrometry (Sequenom Inc, San Diego, CA). Genotyping calls were automatically generated using the MassARRAY TyperAnalyzer software v4.0 (Sequenom Inc, San Diego, CA) and were validated by manual review of the raw mass spectra scatter plots.

Genotyping quality control was performed in samples grouped per country. Samples with $> 4/38$ missing genotypes ($< 89.5\%$ sample success rate) and SNPs with $< 95\%$ genotyping success rate were excluded from further analysis. No SNPs deviated from Hardy-Weinberg equilibrium ($P < 1E-04$), except for a known multiple sclerosis susceptibility SNP in the MHC region in the Norwegian population. Analysis was performed per country with a linear model including gender as covariate for IgG and a logistic model for OCB, followed by a fixed-effects meta-analysis over all countries. An effect was considered replicated when reaching $P < 0.05$ in the replication phase.

Combined analyses

Analysis was performed by a fixed-effects meta-analysis over all cohorts (screening and replication cohorts per country as described before). The percentage of the variance in IgG index explained by variants was calculated by subtracting adjusted r^2 from a full model with

that from the baseline linear model in R. Evidence for interaction between variants, defined as deviation from a multiplicative model, was investigated in a linear (IgG index) or logistic (OCB status) regression in R.

MHC analyses

In the screening phase HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1 genotypes were imputed from SNP data as described previously (Dilthey *et al.*, 2011, The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). The most likely imputed alleles were used as input for testing of association of common HLA alleles with cerebrospinal fluid phenotypes using the same regression approach as described for SNPs above. Independent effects of SNPs/haplotypes in the MHC region were investigated with a likelihood ratio test for OCB status or F test for IgG index comparing a null model and alternative model for phased haplotypes with a minor haplotype frequency of 0.05 including country, seven principal components (screening phase only) and gender as covariates (--chap option in Plink v1.07). The most likely model was obtained by starting with a null model with equal effects for all haplotypes consisting of known and novel replicated MHC signals and allowing separate effects for individual haplotypes, in order of significance, as long as this improved the model with nominal significance.

Multiple Sclerosis Genetic Burden

In the screening phase, a genetic risk score, Multiple Sclerosis Genetic Burden, was calculated on the basis of the number of risk alleles weighted by their effect on multiple sclerosis risk according to the method described previously (Gourraud *et al.*, 2011, Harbo *et al.*, 2014). Two scores were calculated, both including the 57 risk SNPs and either including or excluding alleles imputed as above for the four HLA effects (HLA-DRB1*15:01, DRB1*03:01, DRB1*13:03 and HLA-A*02:01) established in our previous genome-wide association study on susceptibility including this dataset (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). A logistic regression analysis with country as covariate was performed for OCB status, whereas a linear regression analysis including country, gender and seven principal components was performed for IgG index in R.

Results

Clinical and cerebrospinal fluid analyses

The main demographic and clinical characteristics of patients included in the screening and replication phase, stratified by country of origin, are described in Table 1. Among 6,868 multiple sclerosis patients with known OCB status, 6,033 (88%) were OCB positive. The IgG index level in patients recruited from different countries is shown in Supplementary Fig. 1. An elevated IgG index was found in 1,996 of 3,126 patients with known IgG index (64%). IgG index was on average lower in Italian and higher in Danish and Norwegian patients. For a total of 3,044 patients, both OCB status and IgG index were available. Increased IgG index was highly correlated with OCB status ($P < 2E-16$) in the combined dataset (Table 2, Supplementary Fig. 2). Overall, 62% of the multiple sclerosis patients were positive and 10% were negative for both OCB and IgG index (Supplementary Table 1). On average, 26% of the patients were OCB positive but did not have an increased IgG index. An increased IgG index in OCB negative multiple sclerosis patients was very rare (2%).

Gender was highly correlated with both IgG index and OCB status. Women had on average a 1.12-fold higher IgG index ($P = 1.9E-10$) and were 1.3-fold more likely to be OCB positive than men ($P = 9.7E-04$) (Table 2, Supplementary Fig. 3). There was no correlation either

between IgG index or OCB status and the interval between diagnosis and lumbar puncture (median of 1 year). Younger patients had higher IgG indices ($P = 0.0028$), and were more likely to be OCB positive ($P = 0.013$). We found no correlation between multiple sclerosis subtype (bout onset or progressive onset) and IgG index or OCB status after correction for gender and AAO. Patients with a higher IgG index were more severely affected, as assessed by the MSSS ($P = 4.0E-04$).

Genome-wide association screen

After quality control of the screening data, a total of 485,522 SNPs in 3,026 samples and 485,236 SNPs in 938 samples were available for the analysis of OCB status and IgG index, respectively (Supplementary Table 2). Genomic inflation factor was 0.987 for OCB and 1.015 for IgG index (Supplementary Fig. 4).

We first performed a genome-wide analysis of all SNPs and cerebrospinal fluid data available (Fig. 1). Markers in the MHC region on chromosome 6 were associated with OCB status with genome-wide significance ($P < 5E-08$). We identified two regions showing genome-wide significance for association based on the IgG index, the MHC region on chromosome 6 and the IGHC region on chromosome 14. In addition, 14 lead SNPs and two conditional SNPs, and 15 lead SNPs and three conditional SNPs reached suggestive evidence for OCB status and IgG index, respectively (Tables 3 and 4), and were thus taken forward to replication.

When analyzing the 57 multiple sclerosis risk SNPs previously identified in the genome-wide association study (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) (Supplementary Table 3), one SNP (rs12368653) showed nominally significant association with OCB positivity. For IgG index, seven of these 57 SNPs (12%) reached nominal significance. The multiple sclerosis associated risk allele increased the IgG index for five of these SNPs. Since the contribution to the overall multiple sclerosis risk of each of these SNPs is small, we also estimated the Multiple Sclerosis Genetic Burden. This genetic risk score was composed of 57 established non-HLA multiple sclerosis risk SNPs and four classical HLA alleles (Gourraud *et al.*, 2011, The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). A higher Multiple Sclerosis Genetic Burden including the HLA alleles was associated with increased IgG index [fold change = 1.11 (95% CI: 1.07-1.15), $P = 1.33E-09$] and OCB status [OR = 1.51 (95% CI: 1.32-1.74), $P = 3.44E-09$], whereas these associations were substantially reduced when considering only non-HLA risk SNPs [IgG index: fold change = 1.06 (95% CI: 1.00-1.13), $P = 0.038$; OCB: OR = 1.19 (95% CI: 0.96-1.48), $P = 0.12$], indicating the MHC region as the main contributor.

Subsequently, we investigated SNPs known from the literature to be associated with the presence of antibodies in diverse other tissues (Pubmed and Catalogue of Genome-wide Association Studies 4/12/2012) (Supplementary Table 4). A SNP tagging HLA-DRB1*15:01 (rs3129934), which is reported to be protective against IgA deficiency, increased IgG index ($P = 1.37E-07$) as well as the risk of OCB positivity in multiple sclerosis ($P = 3.7E-04$).

Replication

A total of 42 SNPs were taken forward to the replication phase and 38 SNPs were successfully genotyped (Supplementary Table 5). After quality control (Supplementary Table 6), a total of 3,478 patients with OCB status and 2,072 with IgG index data available were analyzed. The same two regions that were associated with genome-wide significance with IgG index in the screening phase, i.e. the MHC and IGHC region, reached genome-wide significance in the replication phase as well (Tables 3 and 4). One novel non-MHC region (rs9807334 near *ELAC1* - *SMAD4*) was replicated with nominal significance for association with OCB status.

Combined analyses

Given the highly significant correlation between OCB status and IgG index, we compared results from a detailed analysis of the MHC class II region for both characteristics (Supplementary Table 7 and Fig. 2). The most likely model in the combined analysis included two independent effects in the MHC class II region on OCB status; a major effect of the rs9271640*A-rs6457617*G haplotype and an additional effect of rs3957148 (tagging SNP with $r^2 = 0.63$ for rs34083746 identified in the screening phase). The first signal is highly correlated with the HLA-DRB1*1501 haplotype ($r^2 = 0.94$), whereas the second is strongly correlated with HLA-DQA1*0301 (rs34083746: $r^2 = 0.93$, rs3957148: $r^2 = 0.64$), and more modestly with both HLA-DRB1*0401 (rs34083746: $r^2 = 0.39$) and *0404 (rs34083746: $r^2 = 0.26$). Carrying the MHC SNP allele associated with OCB positive status at these loci, was associated with a two-fold higher likelihood of being OCB positive than carrying the allele associated with OCB negative status at all three loci [OR = 2.25 (95% CI 1.84-2.75), $P = 8.88\text{E-}16$] (Fig. 2A). The rs9271640*A-rs6457617*G haplotype, correlated with DRB1*1501, was associated not only with OCB status, but also IgG index ($P = 1.59\text{E-}22$) (Fig. 2B). Independent of this haplotype, rs6457617*G had an additional effect increasing IgG index ($P = 3.68\text{E-}06$). Notably, the association of rs6457617 with IgG index was observed in both OCB positive ($P = 4.22\text{E-}10$) and OCB negative multiple sclerosis patients ($P = 6.12\text{E-}04$). SNP rs6457617 is only modestly associated with any classical HLA allele ($r^2 < 0.30$). SNP rs34083746/rs3957148, correlated with DQA1*0301 and associated with OCB negative status, did not appear to influence IgG index levels. No interaction amongst the associated SNPs in the MHC region or between these and the SNP in the IGHC region was observed (data not shown).

The previously reported association of IgG index with SNPs in the IGHC region was replicated as highly significant in the combined dataset ($P = 3.79\text{E-}37$) and explains 4.7 % of the variance in IgG index in the entire dataset. The MHC and IGHC region, the major determinants of variation in IgG index, together explain 7.75% of variance and combined with gender and country, account for 12.65% of variance in IgG index throughout Europe. Forest plots for all association signals per country are given in Supplementary Fig. 5 and 6.

We also investigated the correlation between the main four established genetic effects and AAO, disease course and MSSS with covariates gender and, for the latter two, AAO. After correcting for multiple testing, the A-allele of rs9271640 (that was associated with increased IgG levels and likelihood of OCB positive status), was associated with lower AAO ($P_{\text{screen}} = 4.44\text{E-}04$, $P_{\text{replication}} = 0.082$, $P_{\text{combined}} = 5.68\text{E-}04$). In contrast, at the IGHC locus, the allele that increased IgG index showed association with a later AAO ($P_{\text{screen}} = 0.011$, $P_{\text{replication}} = 0.0066$, $P_{\text{combined}} = 8.23\text{E-}05$) (Supplementary Fig. 7).

Discussion

This study is the largest to date that investigates genetic differences, clinical and demographical characteristics in relation to OCB status (N=6,868) and IgG index (N=3,126) in multiple sclerosis. Our findings strongly support that multiple sclerosis patients with and without OCBs and/or increased IgG index are genetically distinct. This has previously been suggested in other, smaller studies (Kikuchi *et al.*, 2003, Imrell *et al.*, 2006, Idiman *et al.*, 2009, Wu *et al.*, 2009, Romero-Pinel *et al.*, 2011, Buck *et al.*, 2012, Leone *et al.*, 2013, Mero *et al.*, 2013, Yoshimura *et al.*, 2014) and is now confirmed. In accordance with our previous observations and those of others (Lechner-Scott *et al.*, 2011, Mero *et al.*, 2013, Stangel *et al.*, 2013), multiple sclerosis patients with high cerebrospinal fluid antibody levels, as characterized by OCB positive status and/or high IgG index, more often are women and seem to have a lower AAO and higher MSSS. We find that the two cerebrospinal fluid parameters included in this study, OCB status and IgG index, are highly correlated (Mayringer *et al.*, 2005, Link and Huang, 2006, Rinker *et al.*, 2007), supporting that these measurements reflect the same immunological process.

Recently, the IGHC locus on chromosome 14 was reported to be associated with IgG index in Belgian and German multiple sclerosis patients (Buck *et al.*, 2012). This is convincingly replicated in the present extended study with the finding of a strong association of rs11621145 to IgG index ($P_{\text{combined}} = 3.79\text{E-}37$). In addition, we identify two novel association signals in the MHC region as major genetic determinants for IgG index; rs9271640 which is correlated with the HLA-DRB1*15:01 allele, and rs6457617 which is located near the HLA-DQA1 gene but not known to tag any conventional HLA-allele. An interaction between HLA and IGHC has been suggested (Pandey, 2013); however we do not find any evidence to support this hypothesis. Together, these three SNPs from the MHC and IGHC regions explain approximately 7.75 % of the variance in IgG index.

Several studies have suggested that OCB positive and OCB negative multiple sclerosis patients are associated with different HLA-DRB1 alleles (Kikuchi *et al.*, 2003, Imrell *et al.*, 2006, Idiman *et al.*, 2009, Wu *et al.*, 2009, Romero-Pinel *et al.*, 2011, Leone *et al.*, 2013, Mero *et al.*, 2013, Yoshimura *et al.*, 2014). We observe a significant association of SNP rs9271640 tagging HLA-DRB1*15:01 with OCB status and a high correlation of the Multiple Sclerosis Genetic Burden including HLA risk alleles with OCB status. Our findings hence support previous observations of the major established multiple sclerosis risk allele, HLA-DRB1*15:01, being more strongly associated with OCB positive multiple sclerosis than OCB negative multiple sclerosis. The present study also detects a significant difference between OCB positive and negative patients with regard to rs34083746. This SNP is highly correlated with the DQA1*0301 allele shared between DRB1*04 haplotypes. Hence, our findings are consistent with several HLA-DRB1*04 alleles having been shown to increase risk for OCB negative multiple sclerosis, but not OCB positive multiple sclerosis, in previous studies from Europe and Japan (Kikuchi *et al.*, 2003, Imrell *et al.*, 2006, Mero *et al.*, 2013, Yoshimura *et al.*, 2014). The combination of variation in the MHC region has a major impact on the OCB status of multiple sclerosis patients, resulting in a more than two-fold difference in the odds of being OCB positive. In addition to replicating previously suggested associations to OCB status within the MHC region, our analyses provide evidence for a novel locus to be associated with OCB status, rs9807334 near the *ELAC1/SMAD4* genes. The *SMAD4* gene, a signal transduction protein in the tumor growth factor beta pathway, has previously been implicated in class switch recombination and in Experimental Autoimmune Encephalomyelitis and multiple sclerosis (Park *et al.*, 2005, Meoli *et al.*, 2010, Huss *et al.*, 2011) and the same allele has previously been suggested as associated with vaccine response (Ovsyannikova *et al.*, 2012).

The cerebrospinal fluid phenotype association signals in the MHC region we observe have been associated with susceptibility and antibody levels in other diseases. The HLA-DRB1*1501-DQB1*0601 haplotype has been associated with either the presence or increased quantity of immunoglobulins of the IgG, IgA and IgM families both in healthy controls and in disease, including total immunoglobulins (Ferreira *et al.*, 2010), antibodies induced by viruses such as Epstein-Barr virus (Rubicz *et al.*, 2013), and autoantibodies in type 1 diabetes (Ishii *et al.*, 2005) and Sjögren syndrome (Gottenberg *et al.*, 2003) though an opposite correlation is seen for few other antibody responses (Sundqvist *et al.*, 2014). HLA-DQA1*0301, highly correlated with SNP rs34083746, is associated with autoantibody negative disease in ketosis-prone diabetes (Oak *et al.*, 2014). SNP rs6457617 is a major susceptibility factor in rheumatoid arthritis and systemic sclerosis, independent of classical HLA alleles (The International MHC and Autoimmunity Genetics Network, 2009, Radstake *et al.*, 2010, Allanore *et al.*, 2011, Orozco *et al.*, 2014). Of note, the risk allele for these autoimmune diseases decreases the IgG levels in multiple sclerosis. The IGHC locus, on the other hand, has not been associated with antibody levels in other diseases as yet. Overall, the MHC region may enhance antibody production and OCB positive status, whereas the IGHC region has been proposed to influence clearance of antibodies from the cerebrospinal fluid and hence immunoglobulin levels (Buck *et al.*, 2012).

Other studies have shown that IgG levels correlate with the number of B cells, more particularly the number of plasmablasts, in the cerebrospinal fluid (Rudick *et al.*, 1999, Cepok *et al.*, 2001, Cepok *et al.*, 2005). B cell follicles in the meninges have been found in a subset of multiple sclerosis patients and are suggested to play a pathogenic role (Magliozzi *et al.*, 2007). Moreover, a prognostic potential of findings in the cerebrospinal fluid has been proposed, since positivity for OCB is reported to double the risk of developing clinically definite multiple sclerosis in clinically isolated syndrome patients (Tintore *et al.*, 2008). Also, some studies suggest that the inflammation in the brain reflected by cerebrospinal fluid alterations may correlate with neurodegeneration and disease progression (Stangel *et al.*, 2013). Our findings of a lower AAO and a higher MSSS in the multiple sclerosis patients with marked cerebrospinal fluid antibody levels support this hypothesis. In our study, HLA-DRB1*15:01 increases cerebrospinal fluid antibody levels and lowers AAO, as reported previously (Hensiek *et al.*, 2002, The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). However, at the IGHC locus, the allele increasing IgG index appears correlated with higher AAO, indicating that the mechanisms underlying the correlation between genetic determinants, cerebrospinal fluid antibody levels and clinical outcome are not fully understood as yet.

The present study is well-powered, and the clinical characteristics of the patients included support that they are representative for multiple sclerosis patients in general. On average, OCB positivity was found in 88 % and an elevated IgG index in 64 % of patients, in accordance with earlier studies (Dobson *et al.*, 2013, Stangel *et al.*, 2013). Lumbar puncture is routinely performed as a part of the diagnostic process in most of the countries included, but a possible selection bias must be kept in mind when interpreting the clinical findings. OCBs and/or increased IgG index in the cerebrospinal fluid are clinically important hallmarks in multiple sclerosis, and in this large study we show that these disease phenotypes are associated with both genetic variants and clinical and demographic characteristics. The presence of intrathecal immunoglobulin M has been reported as an additional cerebrospinal fluid characteristic in multiple sclerosis patients that may be associated with unfavourable outcome or aggressive disease (Stangel *et al.*, 2013). The study of genetic factors underlying immunoglobulin M levels is currently hampered by the availability of data but will be of future interest.

In summary, in this large study including 6,950 multiple sclerosis patients, we confirm that genetic variants in the immunologically important regions of MHC and IGHC influence OCB status and IgG index in multiple sclerosis patients.

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Figure Legends

Fig. 1. Manhattan plot of genome-wide association screen for A. OCB status and B. IgG index.

Red line indicates genome-wide significance, blue line suggestive evidence.

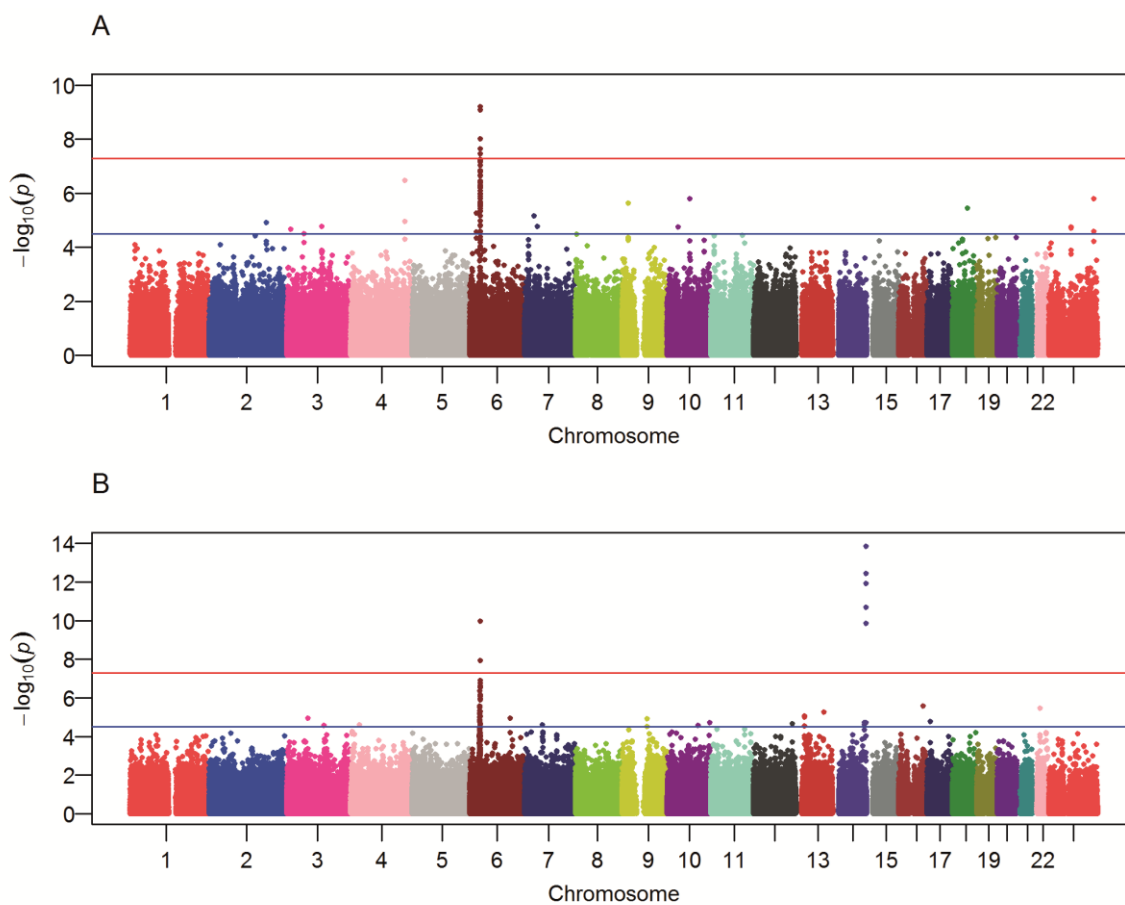
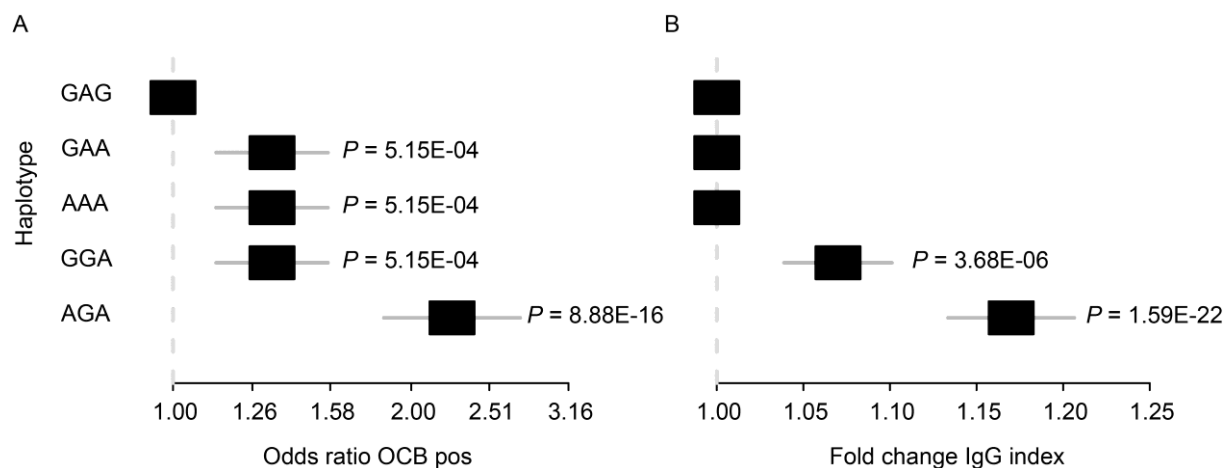


Fig. 2. Most likely model for MHC class II haplotypes (composed of SNPs rs9271640-rs6457617-rs3957148) and cerebrospinal fluid phenotype in the combined analysis: A. OCB status and B. IgG index. GAG haplotype is correlated with HLA-DQA1*0301 ($r^2 = 0.64$), AGA haplotype with HLA-DRB1*1501 ($r^2 = 0.94$). *P* values are given compared to reference haplotypes.



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